Phosphatidylinositol 4,5-bisphosphate anchors cytosolic group IVA phospholipase A_2 to perinuclear membranes and decreases its calcium requirement for translocation in live cells

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Abstract

The eicosanoids are centrally involved in the onset and resolution of inflammatory processes. A key enzyme in eicosanoid biosynthesis during inflammation is Group IVA phospholipase A_2 (also known as cytosolic phospholipase $A_2\alpha$, $cPLA_2\alpha$). This enzyme is responsible for generating free arachidonate (AA) from membrane phospholipids. $cPLA_2\alpha$ translocates to perinuclear membranes shortly after cell activation, in a process that is governed by the increased availability of intracellular Ca^{2+}. However, $cPLA_2\alpha$ also catalyzes membrane phospholipid hydrolysis in response to agonists that do not mobilize intracellular Ca^{2+}. How $cPLA_2\alpha$ interacts with membranes under these conditions is a major, still unresolved issue. Here we report that phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) promotes translocation of $cPLA_2\alpha$ to perinuclear membranes of intact cells in a manner that is independent of rises in the intracellular Ca^{2+} concentration. PtdIns(4,5)P_2 anchors the enzyme to perinuclear membranes and allows for a proper interaction with its phospholipid substrate to release arachidonate.

Introduction

It is well established that the hydrolytic attack of AA-containing phospholipids by cPLA$_2\alpha$ constitutes the first regulatory step of the eicosanoid biosynthetic cascade (Bonventre, 2004; Hirabayashi et al., 2004). cPLA$_2\alpha$ is unique among all the phospholipase A$_2$ family members (Six and Dennis, 2000) due to its substrate selectivity for AA-containing phospholipids and its tight regulation by Ca^{2+} and phosphorylation (Clark et al., 1991; Lin et al., 1993; Gijón et al., 2000a; Dessen et al., 1999). Free AA generated by activated cPLA$_2\alpha$ can be oxygenated into a variety of compounds, called the eicosanoids, that not only are involved in acute and chronic inflammatory diseases such as arthritis, asthma, and postischaemic tissue-injury, but can also regulate physiological processes such as renal function, and female reproductive events including parturition (Bonventre, 2004; Hirabayashi et al., 2004; Uozumi et al., 1997; Bonventre et al., 1997).

cPLA$_2\alpha$ is a cytosolic enzyme in resting cells and translocates to membranes when cell activation takes place. The enzyme possesses a calcium-binding domain (C2) in its N-terminal half that helps the enzyme interact with membrane phospholipid when the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) rises. A single mutation in this domain, i.e. substitution of Asp-43 with Asn, D43N) completely abrogates the Ca^{2+}-dependent translocation of cPLA$_2\alpha$ to cellular membranes and, as a result, AA release is inhibited (Perisic et al., 1999; Gijón et al., 1999; Qiu et al., 1998).

However, stimuli that are known not to affect $[Ca^{2+}]i$ such as bacterial lipopolysaccharide, phorbol esters or okadaic acid, are nonetheless able to induce robust AA mobilization and eicosanoid production responses in a cPLA$_2\alpha$-dependent manner (Shinohara et al., 1999; Gijón et al., 2000b). The mechanism by which

cPLA$_2\alpha$ interacts with membranes under conditions that do not involve increase of intracellular Ca^{2+} concentrations ([Ca^{2+}]i) has remained unaddressed.

In vitro activity studies carried by Leslie and Channon (1990) in the early 90's utilizing a partially purified cPLA$_2\alpha$ preparation, had suggested that anionic phospholipids such as PtdIns(4,5)P_2 and phosphatidic acid, when incorporated into the vesicle substrate, were capable of stimulating the activity of the enzyme and decreasing its Ca^{2+} requirement from mM to nM levels. More recently, Mosior et al. (1998) described the potent and specific increase in affinity of pure cPLA$_2\alpha$ for surfaces containing PtdIns(4,5)P_2 at physiological concentrations, and this effect paralleled an increase in substrate hydrolysis. Importantly, the enhancing effects of PtdIns(4,5)P_2 were observed even in the presence of EGTA ([free Ca^{2+}] < 2 nM). Das and Cho (2002) recently described a cluster of cationic residues in the catalytic domain of cPLA$_2\alpha$ (Lys^{488}, Lys^{541}, Lys^{543}, and Lys^{544} in the human sequence) that appears to play a role in the in vitro activation of the enzyme by PtdIns(4,5)P_2, a finding that was corroborated later on by Six and Dennis (2003).

With regard to intact cells, we observed that in UV light-treated macrophages, increased PtdIns(4,5)P_2 synthesis occurs at resting Ca^{2+} levels, and this parallels the cPLA$_2\alpha$-dependent mobilization of AA (Balsinde et al., 2000). These data led us to propose that PtdIns(4,5)P_2 might be involved in cPLA$_2\alpha$ activation at Ca^{2+} levels equaling those pertaining to unstimulated cells (Balsinde et al., 2000). In this paper we demonstrate that PtdIns(4,5)P_2 anchors cPLA$_2\alpha$ to perinuclear membranes of intact cells in a manner that is independent of rises in [Ca^{2+}]i, thus allowing for proper interaction of the enzyme with its phospholipid substrate to optimally release AA.

Results and Discussion

We were interested in examining whether cellular increases in PtdIns(4,5)P_2 regulate cPLA$_2\alpha$ translocation to intracellular membranes in live cells. To this end, we prepared HEK cells stably expressing a fusion protein of enhanced green fluorescent protein (EGFP) and human cPLA$_2\alpha$. The cells were studied under the confocal microscope during treatment with fluorescent PtdIns(4,5)P_2 (BODIPY^{R}TR-tagged PtdIns(4,5)P_2; abbreviated as TR-PI(4,5)P_2). When complexed with histone or neomycin carriers that counteract the negative charges of TR-PI(4,5)P_2 and make it membrane-permeable (Ozaki et al., 2000), it takes 5-10 min for the phospholipid to penetrate into the cell (Fig. 1). Once inside, TR-PI(4,5)P_2 localized primarily to perinuclear membranes, in accord with previous observations (Ozaki et al., 2000). Movement of TR-PI(4,5)P_2 to perinuclear membranes was closely followed by the complete translocation of cPLA$_2\alpha$ to the same location (Fig. 1A). When EGFP-cPLA$_2$ transfected cells were treated only with the carrier, EGFP-cPLA$_2$ did not translocate (Fig. 1B). Furthermore, when cells transfected with EGFP alone were exposed to TR-PI(4,5)P_2, the green fluorescence pattern did not change (Fig. 1C). AA mobilization was studied under these conditions. Figure 1D shows that exposure of the cells to TR-PI(4,5)P_2 enhanced AA release, thus indicating that membrane phospholipid hydrolysis follows from translocation of cPLA$_2$ to perinuclear membranes.

A possible explanation for TR-PI(4,5)P_2-induced cPLA$_2\alpha$ membrane translocation and subsequent activation could be that the phosphoinositide, once inside the cell, is hydrolyzed by phospholipase C to generate inositol 1,4,5-trisphosphate which results in elevated [Ca^{2+}]i and hence cPLA$_2\alpha$ translocation. However, cPLA$_2\alpha$ translocation to membranes was also observed when the cells were treated with TR-PI(3,4)P_2, whose hypothetical hydrolysis by phospholipase C

would not yield inositol 1,4,5-trisphosphate (Fig. 1E). That both PtdIns(4,5)P_2 and PtdIns(3,4)P_2 isomers modify the subcellular localization of cPLA$_2\alpha$ suggests a charge effect rather than the result of a metabolic transformation via phospholipase C. The PtdIns(3,4)P_2–induced translocation of cPLA$_2\alpha$ was accompanied by an increased release of AA, similar in extent to that produced by PtdIns(4,5)P_2 (data not shown). This is consistent with the in vitro studies of Six and Dennis (2003) showing increased cPLA$_2\alpha$ activity in response to PtdIns(3,4)P_2. Interestingly, Das and Cho (2002) failed to observe an effect of PtdIns(3,4)P_2 on the activity of cPLA$_2\alpha$. The reasons for this discrepancy may likely arise from differences in the experimental protocols followed by these authors.

Experiments were also conducted with the macrophage cell line RAW 264.7. Owing to their immunoinflammatory nature, RAW 264.7 macrophages possess a robust machinery for eicosanoid biosynthesis and thus provide a more appropriate cell context to validate cPLA$_2\alpha$ translocation and AA release in response to PtdIns(4,5)P_2. Exposure of EGFP-cPLA$_2\alpha$-transfected macrophages to TR-PI(4,5)P_2 resulted in membrane translocation of the enzyme (Fig. 2A) and enhanced AA release (Fig. 2B).

We also performed measurements with pure PtdIns(4,5)P_2, isolated from bovine brain. Probably because of the presence of long chain fatty acids in pure PtdIns(4,5)P_2, it was not possible for us to deliver this phospholipid into the cell's interior by using a shuttle. Thus, for these experiments we utilized digitonin-permeabilized cells (Balsinde et al., 2000). Using this procedure, we confirmed that native PtdIns(4,5)P_2 induced translocation of EGFP-cPLA$_2\alpha$ to perinuclear membranes in a similar fashion to that previously observed with BODIPY-TR-PI(4,5)P_2 in Fig. 1 (data not shown).

Mosior et al. (1998) have shown that binding of cPLA$_2\alpha$ to phospholipid vesicles enriched in PtdInsP$_2$ is essentially a Ca^{2+}-independent process. To verify whether this is also the case in live cells, we incubated the cells in a Ca^{2+}-free EGTA-containing buffer. The continued presence of cells in Ca^{2+}-free buffer lowers the intracellular Ca^{2+} level well below that normally occurring in resting cells incubated in a regular Ca^{2+}-containining buffer (Di Virgilio et al., 1984). Under these Ca^{2+}-free conditions, no translocation of cPLA$_2\alpha$ was observed after addition of TR-PI(4,5)P$_2$, even though the phosphoinositide penetrated normally into the cell (Fig. 3). Interestingly, if Ca^{2+} was restored to the incubation medium, a very rapid distribution of cPLA$_2\alpha$ to the perinuclear membranes of TR-PI(4,5)P$_2$-treated cells was observed (Fig. 3). These results suggest that, in cells, the PtdInsP$_2$ effect is not Ca^{2+}-independent; rather, a threshold Ca^{2+} is necessary for cPLA$_2\alpha$ to translocate to membranes. This view is consistent with previous data by Das and Cho (2002).

To further characterize this Ca^{2+} effect, we conducted experiments with a mutant construct where the cPLA$_2\alpha$ has the mutation D43N (EGFP-D43N-cPLA$_2$) which severely limits the ability of the enzyme to bind calcium (Bittova et al., 1999; Perisic et al., 1999; Gijón et al., 1999). As shown in Fig. 4, the mutant EGFP-D43N-cPLA$_2$ did not translocate in response to TR-PI(4,5)P$_2$, even in the presence of 1.3 mM $CaCl_2$ in the incubation buffer, demonstrating that a functional C2 domain is necessary for cPLA$_2$ translocation to perinuclear structures in response to PtdInsP$_2$.

Since TR-PI(4,5)P$_2$ and cPLA$_2\alpha$ appear to move to similar intracellular structures (Figs. 1 to 3), a co-localization analysis was performed. Fig. 5A shows the merge of green (from the EGFP-cPLA$_2$) and red (from the TR-PI(4,5)P$_2$) fluorescences in cells treated for 20 min with TR-PI(4,5)P$_2$, suggesting co-localization in the perinuclear region. Analysis of co-localization with the LaserPix confocal image

software (BioRad) provides a better definition of the pixels showing perfect co-localization of both fluorescences (Fig. 5B, white mask). In Fig, 5C, a three-dimensional representation of such co-localization is shown, and the color of each pixel defines the level of co-localization. Nuclear membrane and perinuclear structures (most likely Golgi and endoplasmic reticulum) show the most intense co-localization. As a control for the co-localization analyses, note a cell in the bottom left corner of Figs 5A-C that does not express EGFP-cPLA$_2\alpha$ and thus stains red, and another cell in the bottom right corner that expresses the EGFP-cPLA$_2\alpha$ but did not take TR-TR-PI(4,5)P$_2$ in, and thus stains only green. Accordingly, these two cells do not show in the co-localization mask (Fig. 5B) or in the three-dimensional representation (Fig. 5C).

Taking advantage of the fact that in the absence of extracellular calcium, TR-PI(4,5)P$_2$ fails to induce EGFP-cPLA$_2\alpha$ translocation to membranes (see above), green and red fluorescences can be analyzed in the same cell at different localizations (Fig. 5D). Under these conditions, the colocalization coefficients obtained with the LaserPix software were: red to green, 0.37 ± 0.08, and green to red, 0.33 ± 0.06. After restoring calcium to the extracellular medium, the co-localization analysis was repeated on the same cells and the data were: red to green, 0.40 ± 0.05; and green to red, 0.88 ± 0.01.

Altogether, the results presented so far indicate that PtdIns(4,5)P$_2$ functions to anchor cPLA$_2$ to the perinuclear and nuclear membranes in live cells, by a process that requires the presence of a certain threshold level of Ca^{2+} as well as a functional Ca^{2+} binding site in the enzyme (Fig. 3). Thus, PtdIns(4,5)P$_2$ may either provide a second binding site for cPLA$_2\alpha$ in addition to the Ca^{2+}-binding site, or act to lower the Ca^{2+} requirement of cPLA$_2\alpha$ for membrane binding. To evaluate these two

possibilities, changes in $[Ca^{2+}]i$ were monitored in Fluo-3-loaded cells exposed to the phosphoinositide (Fig. 6). TR-PI(4,5)P_2 induced changes in $[Ca^{2+}]i$ with a very different pattern, potency and kinetics from the calcium ionophore ionomycin, which is also known to potently induce cPLA$_2\alpha$ membrane translocation (Gijón et al., 2000a) (cf. Figs. 6A and 6B). Given these differences, we proceeded to determine the minimal Ca^{2+} requirements for TR-PI(4,5)P_2 to induce cPLA$_2\alpha$ translocation. To this end, cells were incubated in a Ca^{2+}-free medium, exposed to either TR-PI(4,5)P_2 or ionomycin. Thereafter different $CaCl_2$ concentrations were added to the incubation medium, and confocal microscopy images of the cPLA$_2\alpha$ movement were taken. $[Ca^{2+}]i$ was monitored in Fluo-3-loaded cells in parallel under exact experimental conditions. Figs. 7A and 7B show that TR-PI(4,5)P_2 promoted cPLA$_2\alpha$ membrane translocation at extracellular Ca^{2+} concentrations between 6-30 μM, which translate to 50-220 nM in terms of $[Ca^{2+}]i$. It is particularly striking that TR-PI(4,5)P_2-induced translocation of cPLA$_2\alpha$ begins to be detected in all the cells analyzed already at an extracellular Ca^{2+} concentration of 15 μM, which only induces minimal changes in $[Ca^{2+}]i$ (~50 nM) (Figs. 7A and 7C). Note, on the other hand, that ionomycin promotes visible translocation of cPLA$_2\alpha$ only at higher extracellular Ca^{2+} concentrations, approx. 60-150 μM, which raise $[Ca^{2+}]i$ up to 350-400 nM (Figs. 7B and 7D). These results provide strong evidence that TR-PI(4,5)P_2-induced cPLA$_2\alpha$ translocation occurs at substantially lower $[Ca^{2+}]i$ levels than that triggered by ionomycin.

In vitro mutagenesis studies have identified a cluster of four Lys as the putative PtdInsP_2-binding site (Das and Cho, 2002; Six and Dennis, 2003). In the human enzyme, these are Lys^{488}, Lys^{541}, Lys^{543}, and Lys^{544}. We constructed a mutant in which Lys^{488} was substituted by Glu and the three other Lys by Ala (EGFP-K488E/K541A/K543A/K544A-cPLA$_2\alpha$; EGFP-4KE/A-cPLA$_2\alpha$) (Das and Cho, 2002;

Six and Dennis, 2003). When transfected into cells, the EGFP-4KE/A-cPLA$_2\alpha$ mutant actually translocated in response to TR-PI(4,5)P_2 (Fig. 8A), although the degree of co-localization between mutant enzyme and phospholipid was considerably lower than that previously observed with the wild type enzyme (Fig. 8B). More importantly, cells transfected with the EGFP-4KE/A-cPLA$_2\alpha$ mutant did not mobilize AA in response to TR-PI(4,5)P_2, while cells transfected with the wild-type enzyme did it readily (Fig. 9). These results demonstrate that the four-Lys cluster described by Das and Cho (2002) is indeed crucial for cPLA$_2\alpha$ to translocate to membrane in a functionally-active form. That the EGFP-4KE/A-cPLA$_2\alpha$ mutant also translocates to membrane in response to PtdInsP$_2$ may suggest the existence of additional binding site(s) within the enzyme. However, since membrane binding of the EGFP-4KE/A-cPLA$_2\alpha$ mutant is not productive (i.e. no AA is released), it is probably of very limited biological significance. The behavior of the mutant EGFP-4KE/A-cPLA$_2$ clearly suggests that translocation of the enzyme to membrane does not necessary lead to enhanced AA release.

It has previously been demonstrated that PtdInsP$_2$ does not increase the specific activity of the K541A/K543A/K544A mutant, as measured by an in vitro assay (Das and Cho, 2002; Six and Dennis, 2003). In our intact cell system, PtdInsP$_2$ promotes translocation of the construct EGFP-4KE/A-cPLA$_2$ to perinuclear membranes, but this is not accompanied by an increased AA release, which is in accordance with the aforementioned in vitro studies (Das and Cho, 2002; Six and Dennis, 2003). In these studies an increase in the basal activity of the mutated enzyme as compared to the wild type enzyme was also shown. Our studies in intact cells failed to obtain reproducible evidence for such a phenomenon, as measured by basal AA release. It appears likely that in intact cells, the basal state of the cPLA$_2\alpha$

may be governed by multiple factors, some of which may not be apparent in an in vitro assay.

Our data, along with results from previous studies (Das and Cho, 2002; Six and Dennis, 2003) demonstrate that the positive charge generated by Lys^{488}, Lys^{541}, Lys^{543} and Lys^{544}, allows the $cPLA_2$ to properly bind to $PtdInsP_2$. Mutation of these Lys leads to changes in the specific activity of the enzyme compared to the wild type enzyme, as measured in an in vitro assay (Das and Cho, 2002; Six and Dennis, 2003). We have extended these data by showing decreased AA release in the mutants in response to $PtdInsP_2$. The structure of those four Lys does not conform to the structure of well known phosphoinositide-binding domains such as the plekstrin-homology (PH), the FYVE, or the Phox-homology (PX) domains (Lemmon, 2003). It is likely that this cationic cluster belongs in a novel, yet-undefined phosphoinositide-binding domain, as suggested elsewhere (Das and Cho, 2002).

Based on all the above findings, it is intriguing to speculate with novel modes of $cPLA_2\alpha$ interaction with cellular membranes leading to enhanced phospholipid hydrolysis. By using a methodology that preserves cellular integrity, i.e. shuttling fluorescent $PtdInsP_2$ into the cell by the use of lipophilic carriers, we have studied the interaction between $PtdInsP_2$ and $cPLA_2\alpha$ under a physiologically relevant setting, thus avoiding potential problems arising from the use of cellular fixation or permeabilization techniques. Our live cell studies clearly indicate that variations in the cellular $PtdIns(4,5)P_2$ may help regulate the physical location of $cPLA_2\alpha$. Importantly however, for this regulatory role of $PtdIns(4,5)P_2$ to manifest, an intact C2 domain within the enzyme and a certain threshold level of intracellular calcium are required. The results suggest a sequential mode of $cPLA_2\alpha$ interaction with the membrane, where the enzyme first binds via the C2 domain in the presence of low Ca^{2+} levels.

This is followed by binding to PtdIns(4,5)P_2 via the cationic cluster of four Lys, which positions the enzyme in the proper manner to effect phospholipid hydrolysis and AA release.

In this model of cPLA$_2\alpha$ activation by PtdIns(4,5)P_2, the threshold Ca^{2+} level required is far below that found in stimulated cells, and resembles more that of resting cells, i.e. ~50 nM. This is in contrast with studies in vitro, which reflected no Ca^{2+} requirement for cPLA$_2\alpha$ binding to and hydrolysis of phospholipids in mixed micelles Six and Dennis, 2003). This difference highlights the very complex nature of the interaction of cPLA$_2\alpha$ with a biological membrane in a live cell context. cPLA$_2\alpha$ has been shown to physically interact with ceramide 1-phosphate (Subramanian et al., 2005) and a number of proteins, including vimentin (Nakatani et al., 2000) and a splice variant of Tip60 (Sheridan et al., 2001). Thus it is likely that in cells, numerous entities in addition to PtdInsP_2 may interact with cPLA$_2\alpha$ and modulate its membrane-binding properties.

It is important to note that the above scenario does not exclude the possibility that PtdInsP_2 binding may occur first, and this in turn augments the calcium-dependent lipid binding of the C2 domain. Such a sequence of events has received some experimental support by the recent work of Subramanian et al. (2005) in which ceramide 1-phosphate was found to increase cPLA$_2\alpha$ activity by interacting with the C2 domain, thereby lowering the calcium requirement for translocation.

Our findings provide a mechanistic insight into the intracellular signaling triggered by stimuli like bacterial lipopolysaccharide, UV light, phorbol esters or okadaic acid, which do not mobilize Ca^{2+} from internal stores, yet they all activate the cPLA$_2\alpha$–mediated release of AA (Shinohara et al., 1999; Gijón et al., 2000b). It is likely that the increased synthesis of PtdInsP_2 that occurs during cellular stimulation

by these agonists (Balsinde et al., 2000; Taylor et al., 1984), together with the low nanomolar levels of $[Ca^{2+}]i$, provides the signals to anchor cPLA$_2\alpha$ to perinuclear membranes. Two subfamilies of kinases, termed type I and type II, are involved in the biosynthesis of PtdIns(4,5)P_2 during cell activation. Type I consists of PtdIns(4)P 5-kinases, while type II consists of PtdIns(5)P 4-kinases. Members of both subfamilies have been localized in the perinuclear region of cells (Doghman et al., 2003). On the basis of our findings we propose that PtdIns(4,5)P_2 formed by phosphatidylinositol monophosphate kinases at the perinuclear envelope may help recruit cPLA$_2\alpha$ to this compartment at low $[Ca^{2+}]i$ levels. Future studies should shed additional light on the mechanisms governing PtdIns(4,5)P_2 accumulation in perinuclear membranes and their overall role in cPLA$_2\alpha$-mediated AA signaling.

Materials and Methods

Reagents. Cell culture media and supplements were purchased from Gibco (Paisley, Scotland, UK). BODIPY^{R}-TR-X C_6-PI(4,5)P_2, BODIPY^{R}-TR-X C_6-PI(3,4)P_2, carriers (neomycin and histone), and Fluo-3-AM were purchased from Molecular Probes (Carlsbad, CA, USA). Other reagents were from Sigma Ibérica (Madrid, Spain).

Lipid preparation. Lipids were prepared following the manufacturer's instructions. Briefly, 2 μg phospholipid was mixed with 2 μl carrier (neomycin or histone, 0.5 mM), resuspended in 200 μl HBSS containing 10 mM HEPES and, unless otherwise indicated, 1.3 mM $CaCl_2$, sonicated in a water bath for 2 min, and allowed to rest at 37°C for 10 min before use. Final concentration of PtdInsP_2 in the solution is 5.7 μM.

Cells - HEK cells were cultured in DMEM supplemented with 2 mM glutamine, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37ºC in a 5% CO_2 humidified incubator. Cells were passaged twice a week by trypsinization. Cells (40-70% confluence) were transfected with 1 μg plasmid/ml using Lipofectamine Plus^{TM} (Invitrogen), following the manufacturer's instructions. For stably transfected cells, 1 mg/ml G418 was used for selection and subsequent passages. RAW 264.7 cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37 ºC in a 5% CO_2 humidified incubator.

Constructs. The DNA sequence of human cPLA$_2\alpha$ was cloned into the pEGFP vector (Clontech, Palo Alto, CA) using Hind-III and Pst-I cloning sites. This construct codes for the

expression of a fusion protein containing an *N*-terminal EGFP followed by the entire sequence of the human cPLA$_2\alpha$. Wild type cPLA$_2\alpha$ was mutagenized within the C2 domain by replacing Asp^{43} with Asn (D43N), using the QuikChange XL Site-Directed Mutagenesis kit from Stratagene and the oligonucleotides 5'-CATGCTTGATACTCCAAATCCCTATGTGGAAC-3' (forward) and 5'-GTTCCACATAGGGATTTGGAGTATCAAGCATG-3' (reverse). Mutagenesis was confirmed by sequencing. Wild type EGFP-cPLA$_2\alpha$ was also mutagenized to produce EGFP-D488E/D541A/D543A/D544A-cPLA$_2$ in two steps. First, the mutant EGFP-D541A/D543A/D544A-cPLA$_2$ was obtained by using the QuikChange XL Site-Directed Mutagenesis kit from Stratagene and the oligonucleotides 5´-GAGCCTCTGGATGTCGCAAGTGCAGCGATTCATGTAGTGGACAG-3´ (forward) and 5´-CTGTCCACTACATGAATCGCTGCACTTGCGACATCCAGAGGCTC-3´. Then, a second round of mutagenesis was performed to change Lys^{488} to Glu using the primers: 5´-GGACGTGCTGGGGAGGTACACAACTTCATGC-3´ (forward) and 5´-GCATGAAGTTGTGTACCTCCCCAGCACGTCC-3´(reverse).

Confocal microscopy - Cells were seeded on glass bottom culture dishes (MatTek Corp., Ashland, MA, USA) coated with Poly-L-lysine (Sigma). After 24 hours, the culture medium was replaced with HBSS containing 10 mM HEPES and 1.3 mM $CaCl_2$. Cells were monitored at 37°C by confocal microscopy using a Bio-Rad laser scanning Radiance 2100 system coupled to a Nikon TE-2000U inverted microscope equipped with a DH-35 tissue culture dish heater (Warner Instruments). Images were obtained with a CFI Plan Apo 60X, oil immersion, 1.40 NA objective, which provided a theoretical confocal layer thickness of approximately 0.4 μm at the wavelengths used. Green fluorescence from the EGFP was monitored at 488 nm argon laser excitation and the combination of a HQ500 long band pass and a HQ560 short band

pass blocking filters. Red fluorescence from BODYPI-TRx was monitored at 543 nm HeNe laser excitation using a HQ590/570 long band pass blocking filter. In some experiments cells were incubated with HBSS without calcium and then discrete amounts of $CaCl_2$ were added sequentially to obtain extracellular concentrations of 6, 15, 30, 60, 150, and 300 μM.

AA release - HEK cells (2.5 x 10^5/well) were labeled with 0.5 μCi [^{3}H]AA for 20 h. Cells were then extensively washed and overlaid with 0.5 ml serum-free DMEM supplemented with 0.1 mg/ml of albumin, and treated with 5 μM thimerosal for 15 min to blunt fatty acid reacylation (Pérez et al., 2004). Cells were then treated with $PtdInsP_2$, and the supernatants were collected at different time points. After extraction, lipids were separated by thin-layer chromatography using the system n-hexane/diethyl ether/acetic acid (70:30:1 by volume). Spots corresponding to AA and phospholipid were scraped and radioactivity was quantified by liquid scintillation counting.

Intracellular Ca^{2+} measurements. HEK cells were loaded with 3 μM Fluo-3-AM for 20 min in medium with 10% serum at 37ºC in a 5% CO_2 incubator. Cells were then washed and incubated with HBSS containing 10 mM HEPES and 1.3 mM $CaCl_2$. Fluorescence was monitored under confocal microscope at 488 laser excitation and the combination of a HQ500 long band pass and a HQ560 short band pass blocking filters, having the iris totally open. At the end of each experiment calibration was done as previously described by Kao et al. (1989), using $MnSO_4$ at a final concentration of 2 mM, and lysis with 40 μM saponin to obtain the background signal. Where analysis of intracellular calcium levels was studied in the presence of different concentration of

extracellular $CaCl_2$, cells were loaded with Fluo-3, washed with HBSS with 2 mM EGTA, and layered with HBSS containing 10 mM HEPES in the absence of $CaCl_2$. Different amounts of $CaCl_2$ were added every 10 min, and fluorescence was recorded every 15 seconds under a confocal microscopy. Calibration was also performed at the end of each experiment as indicated above.

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Abbreviations List

AA, arachidonic acid; cPLA$_2\alpha$, cytosolic Group IVA phospholipase A$_2$; [Ca^{2+}]I, intracellular Ca^{2+} concentration; PtdIns, phosphatidylinositol bisphosphate.

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FIGURE LEGENDS

Figure 1. EGFP-cPLA$_2$ translocation and AA release driven by TR-PIP$_2$. EGFP-cPLA$_2$- (A, B, E) or EGFP- (C) transfected HEK cells were treated with TR-PI(4,5)P$_2$/histone (A, C), TR-PI(3,4)P$_2$/histone (E), or carrier only (histone, B). Pictures were taken at the indicated time points under the confocal microscope. D) [^{3}H]AA-labeled HEK cells were either untreated (open circles) or treated with TR-PI(4,5)P$_2$/carrier (closed circles), and [^{3}H]AA release was measured at the indicated time periods. Each experiment was performed at least three times with identical results.

Figure 2. TR-PIP$_2$ effects on RAW 264.7 cells. RAW 264.7 macrophages transfected with EGFP-cPLA$_2$ were treated with TR-PI(4,5)P$_2$/histone for the indicated periods of time and analyzed by confocal microscopy (A) or for [^{3}H]AA release (B) as in Figure 1. Each experiment was performed at least three times with identical results.

Figure 3. Ca^{2+} is necessary for EGFP-cPLA$_2$ translocation in response to TR-PI(4,5)P$_2$. EGFP-cPLA$_2$-transfected HEK cells were treated with TR-PI(4,5)P$_2$/histone in HBSS medium without Ca^{2+}, and pictures were taken under the confocal microscope. After a 30-min treatment, 1.3 mM Ca_2Cl was added to the incubation medium (the two columns on the right hand side), and pictures were taken at the indicated time points. Each experiment was performed at least three times with identical results.

Figure 4. A intact Ca^{2+} binding domain is necessary for EGFP-cPLA$_2$ translocation in response to TR-PI(4,5)P$_2$. HEK cells transfected with the mutant EGFP-D43N-

$cPLA_2$ were treated with TR-$PI(4,5)P_2$/histone in HBSS with 1.3 mM $CaCl_2$ and pictures were taken at the indicated time points by confocal microscopy. Each experiment was performed at least three times with identical results.

Figure 5. EGFP-$cPLA_2$ and TR-$PI(4,5)P_2$ colocalization analysis. HEK cells transfected with the construct EGFP-$cPLA_2$ were treated with TR-$PI(4,5)P_2$ for 20 min in the presence (A) or absence (D) of extracellular calcium, and pictures of green and red fluorescences were taken by confocal microscopy. The merge of both fluorescences is also shown. LaserPix software analysis of co-localization fluorescences in (A) is shown as a white mask (B), or as a colored three-dimensional representation, where red means maximum level of colocalization (255) and dark blue means the minimum (0) (C). Each experiment was performed at least three times with identical results.

Figure 6. Intracellular Ca^{2+} levels in HEK cells. Intracellular measurements of Ca^{2+} were performed in Fluo-3-loaded HEK cells treated with TR-$PI(4,5)P_2$/histone (A) or 5 μM ionomycin (B) in buffer containing 1.3 mM $CaCl_2$. Arrows indicate the time point at which the stimuli were added. Each data point represents the average of 20-40 cells. Fluorescence of Fluo-3 was detected by microscopy and recorded every 15-30 s. Each experiment was performed at least three times with identical results.

Figure 7. Intracellular Ca^{2+} requirements for TR-$PI(4,5)P_2$-driven translocation of EGFP-$cPLA_2$. Fluo-3-loaded HEK cells were first treated with TR-$PI(4,5)P_2$/histone (A) or 5 μM ionomycin (B) in a buffer without Ca^{2+} and later, different $CaCl_2$ amounts were added as indicated. Fluorescence of Fluo-3 was detected by microscopy and

recorded every 15-30 s. Each data point represents the average of 20-40 cells. Translocation of EGFP-cPLA$_2$ was analyzed by confocal microscopy under the same conditions indicated above for HEK cells (C, cells treated with TR-PI(4,5)P$_2$/histone; D cells treated with 5 mM ionomycin). Extracelular concentration of $CaCl_2$ is indicated. Each experiment was performed at least three times with identical results.

Figure 8. Analysis of the EGFP-4KE/A-cPLA$_2$ mutant. (A) HEK cells transfected with the mutant EGFP-4KE/A-cPLA$_2$ were treated with TR-PI(4,5)P$_2$/histone and pictures were taken under the confocal microscope for the indicated time periods. (B) Co-localization analyses (LaserPix software) of the fluorescence from EGFP-4KE/A-cPLA$_2$ mutant and TR-PI(4,5)P$_2$ or EGFP-cPLA$_2$ and TR-PI(4,5)P$_2$ as a control, were performed in the presence of absence of extracellular calcium, as indicated. Co-localization index of red to green (black bars) or green to red (grey bars) is shown. Each data barr represents the average of 10-20 cells. Experiments were performed at least three times with identical results.

Figure 9. AA released by HEK cells transfected with the mutant EGFP-4KE/A-cPLA$_2$. [^{3}H]AA-labeled HEK cells transfected with the EGFP-cPLA$_2$ construct (closed circles) or the mutant EGFP-4KE/A-cPLA$_2$ (open circles) were treated with TR-PI(4,5)P$_2$/carrier and [^{3}H]AA release was measured at the time indicated time points. Each experiment was performed at least three times with identical results.

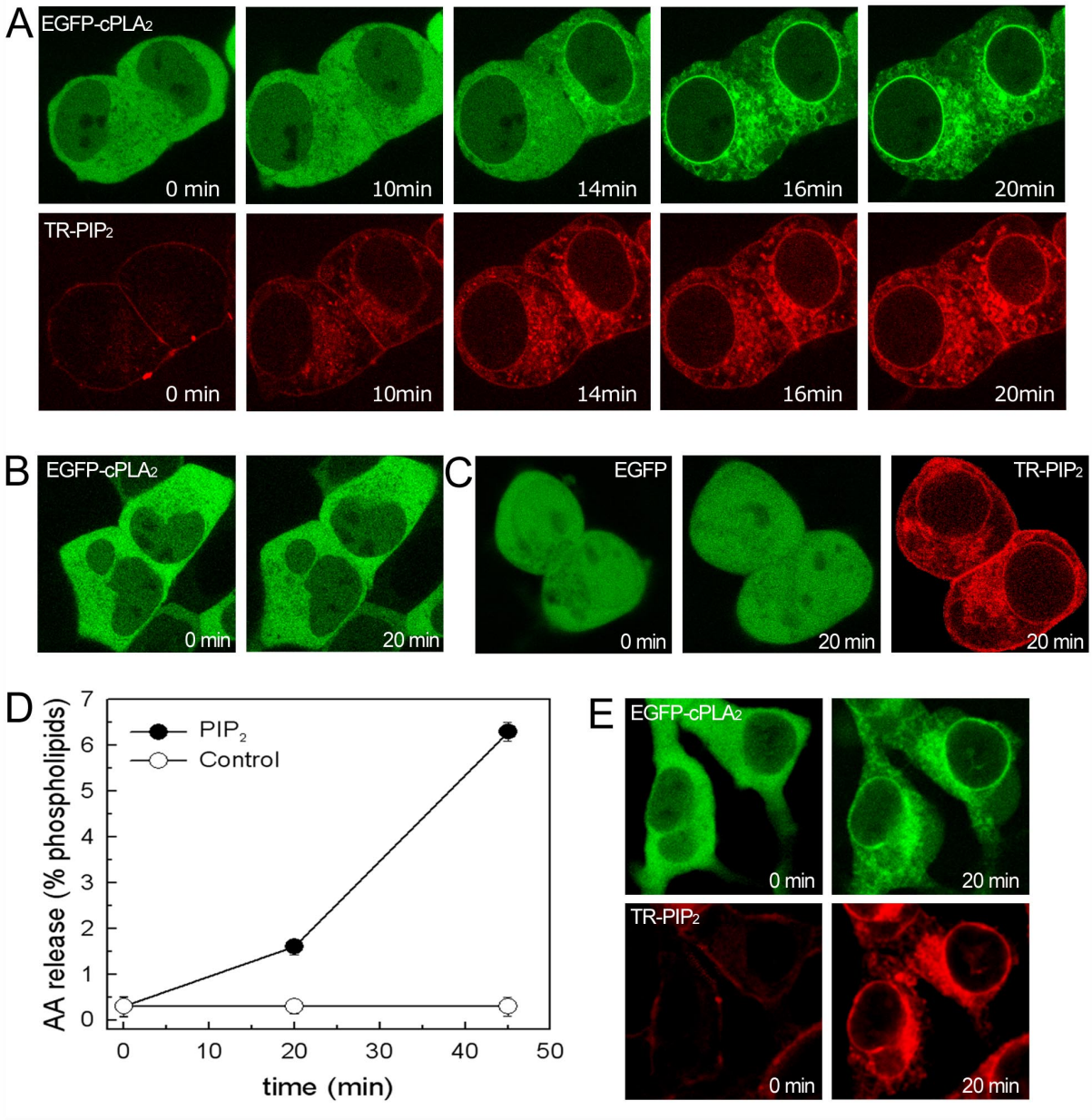
A
EGFP-cPLA2
0 min
10min
14min
16min
20min
TR-PIP2
0 min
10min
14min
16min
20min
B
EGFP-cPLA2
0 min
20 min
C
EGFP
0 min
20 min
TR-PIP2
20 min
D
AA release (% phospholipids)
PIP2
Control
time (min)
E
EGFP-cPLA2
0 min
20 min
TR-PIP2
0 min
20 min

Figure 1

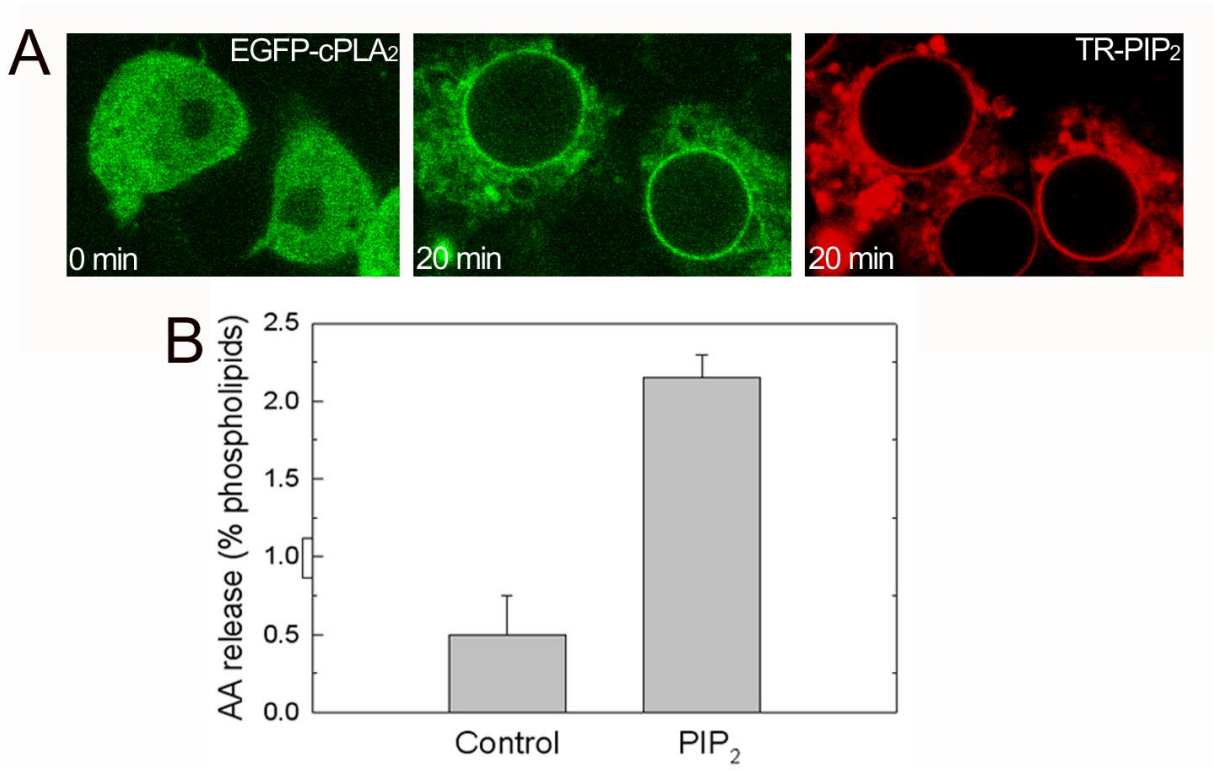
A
EGFP-cPLA2
0 min
20 min
TR-PIP2
20 min
B
AA release (% phospholipids)
2.5
2.0
1.5
1.0
0.5
0.0
Control
PIP2

Figure 2

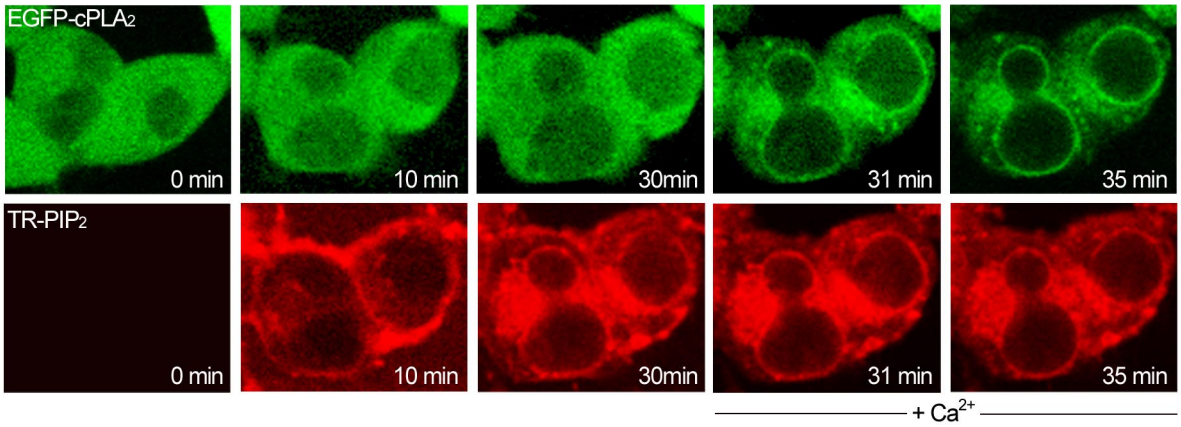
EGFP-cPLA2
0 min
10 min
30min
31 min
35 min
TR-PIP2
0 min
10 min
30min
31 min
35 min
+ Ca2+

Figure 3

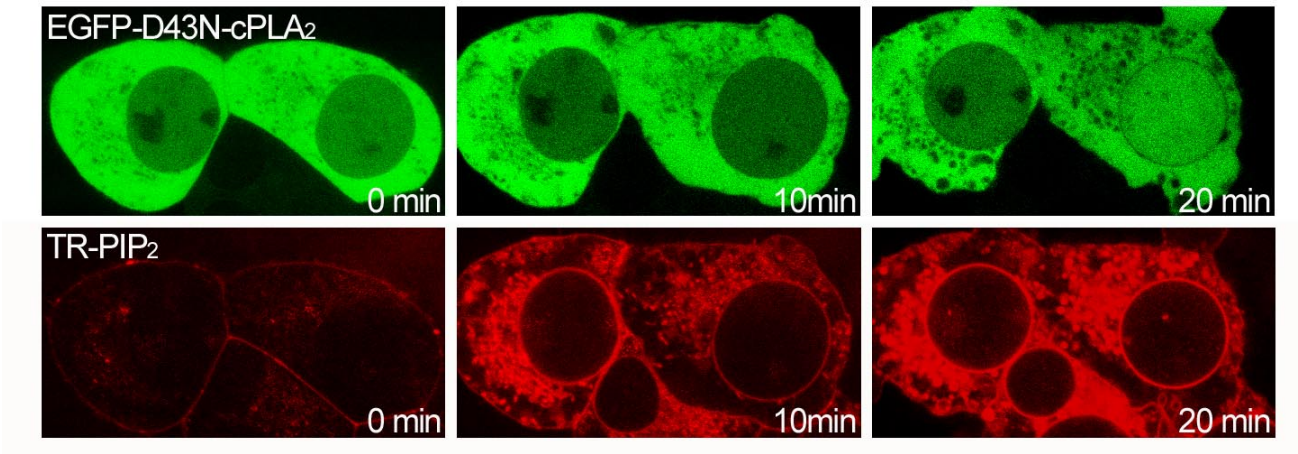

Figure 4

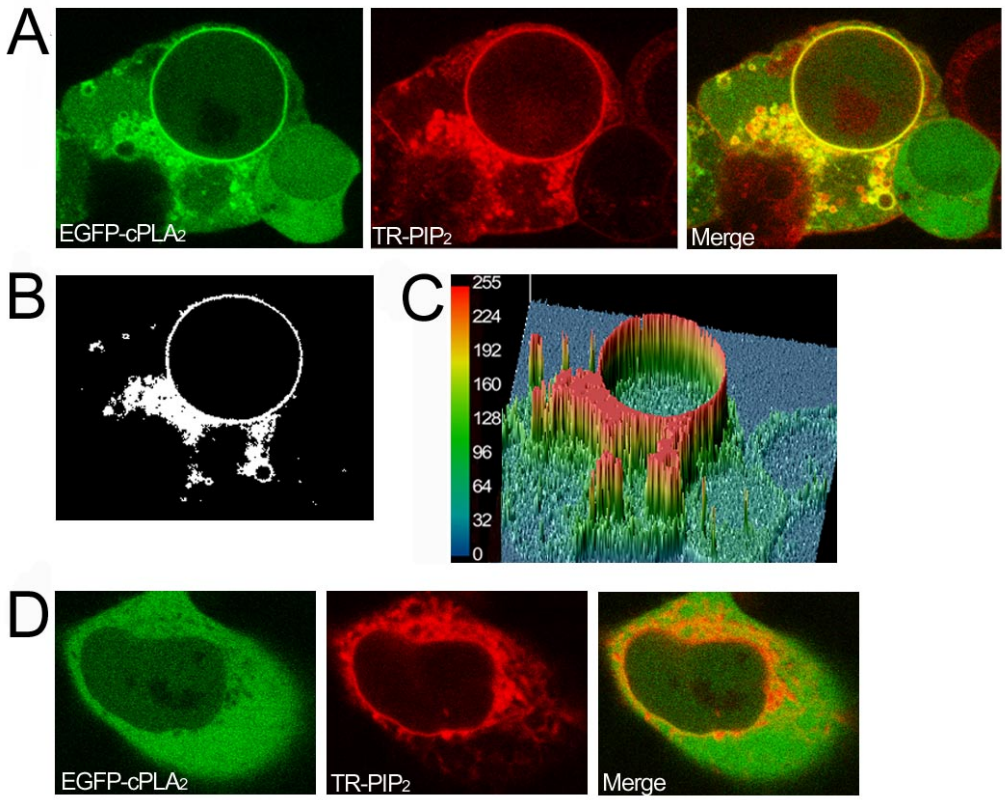
A
EGFP-cPLA2
TR-PIP2
Merge
B
C
255
224
192
160
128
96
64
32
0
D
EGFP-cPLA2
TR-PIP2
Merge

Figure 5

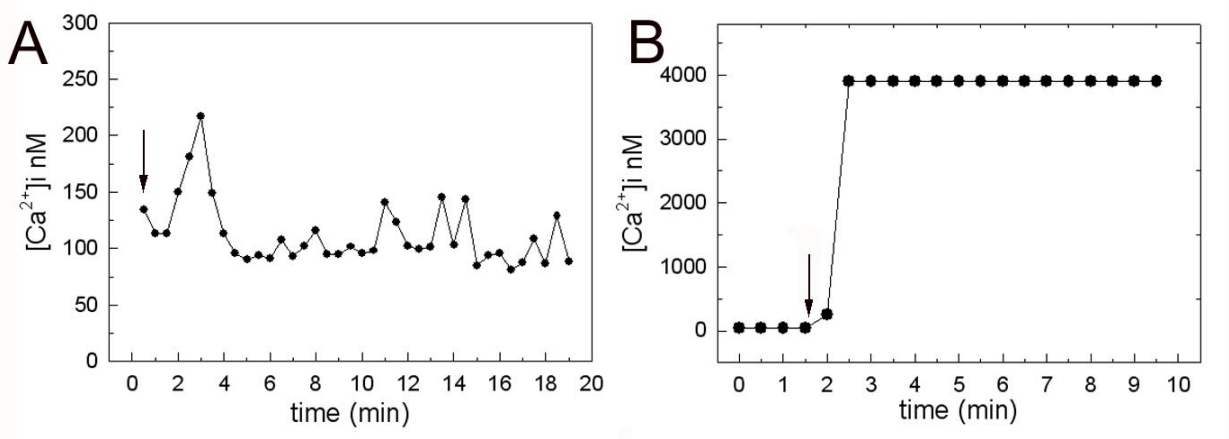

Figure 6

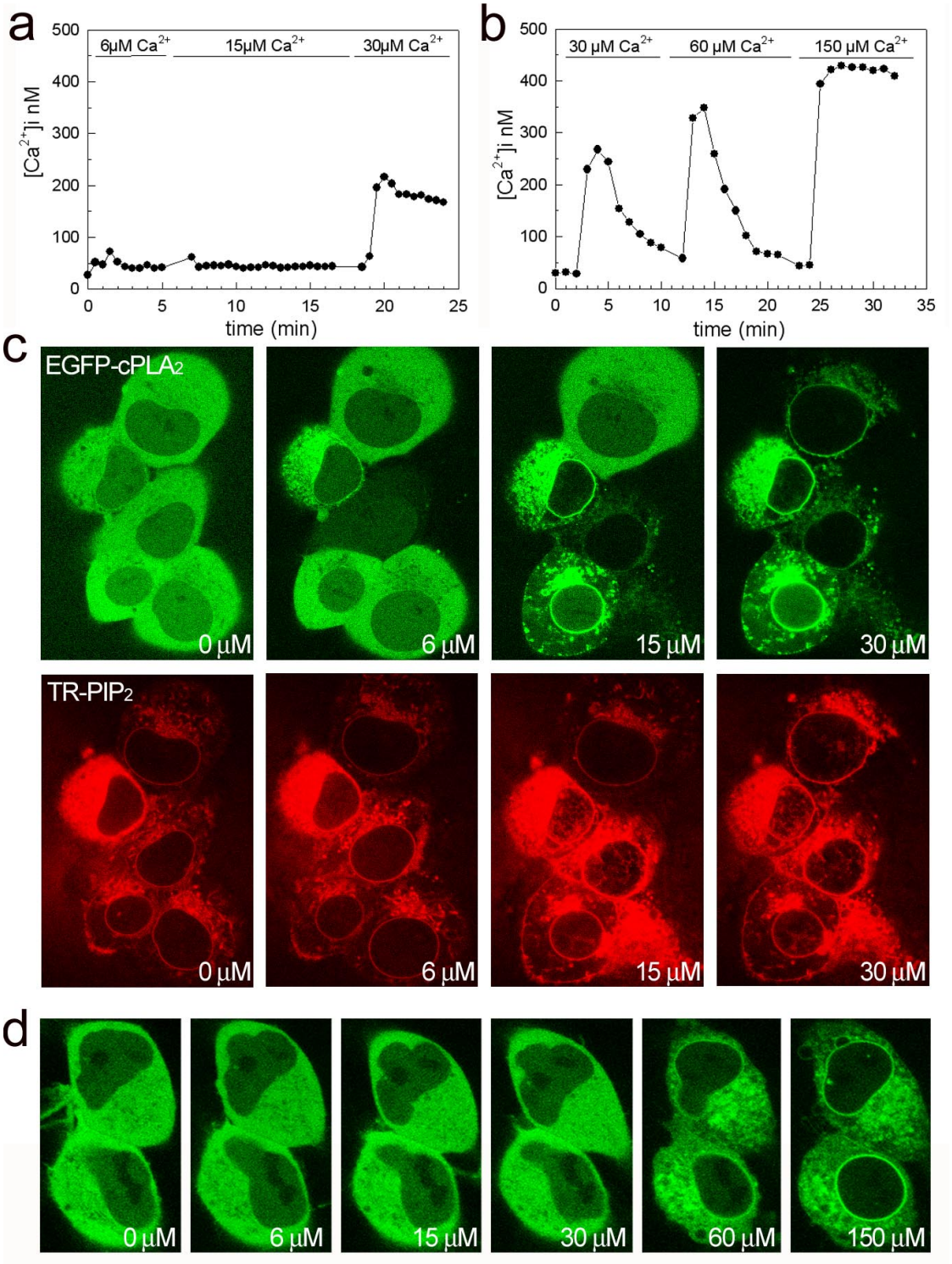
a
6μM Ca2+
15μM Ca2+
30μM Ca2+
[Ca2+]i nM
time (min)
b
30 μM Ca2+
60 μM Ca2+
150 μM Ca2+
[Ca2+]i nM
time (min)
c
EGFP-cPLA2
0 μM
6 μM
15 μM
30 μM
TR-PIP2
0 μM
6 μM
15 μM
30 μM
d
0 μM
6 μM
15 μM
30 μM
60 μM
150 μM

Figure 7

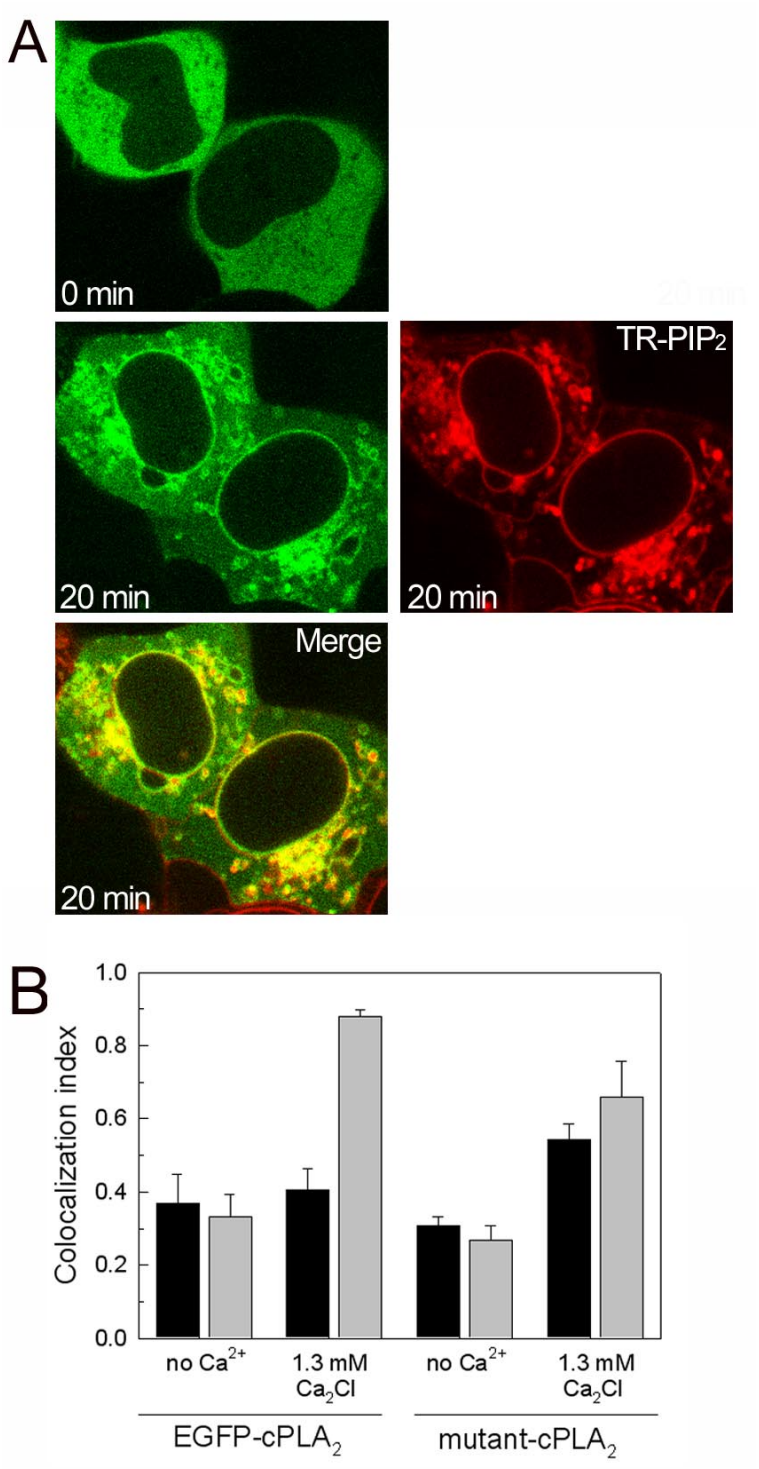
A
0 min
20 min
TR-PIP2
20 min
Merge
20 min
B
Colocalization index
1.0
0.8
0.6
0.4
0.2
0.0
no Ca2+
1.3 mM Ca2Cl
no Ca2+
1.3 mM Ca2Cl
EGFP-cPLA2
mutant-cPLA2

Figure 8

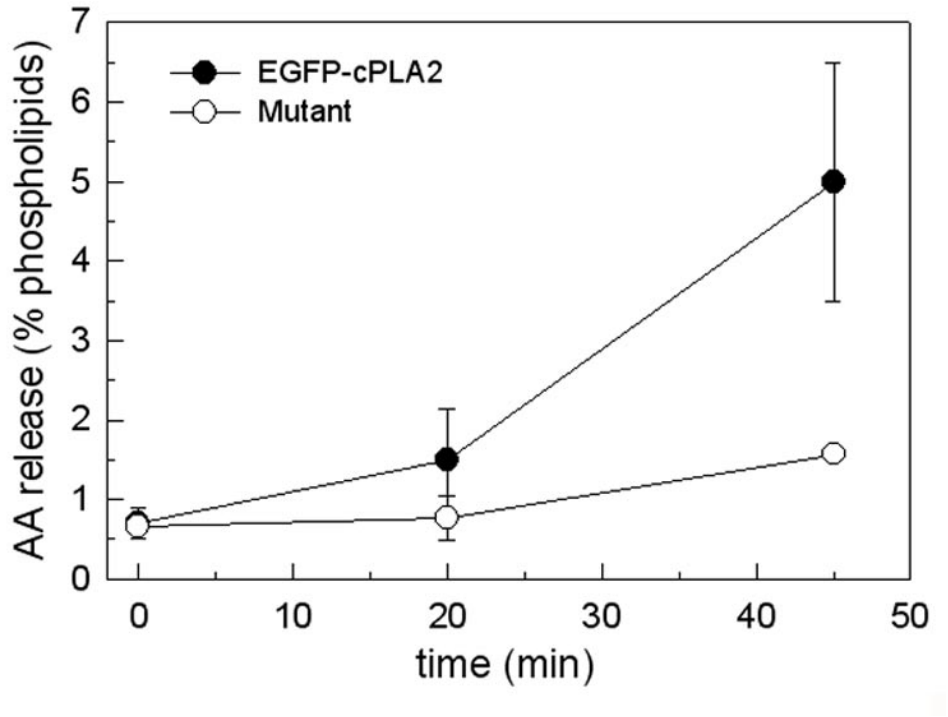
EGFP-cPLA2
Mutant
AA release (% phospholipids)
time (min)
0
1
2
3
4
5
6
7
0
10
20
30
40
50

Figure 9